See, Paper 24, Page 3, Paragraph 5.

Applicants respectfully disagree and traverse.

Applicants reiterate that they need only make *one* credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. § 101 and 35 U.S.C. § 112; additional statements of utility, even if not "credible," do not render the claimed invention lacking in utility. *See, e.g., Raytheon v. Roper*, 220 U.S.P.Q. 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984) ("When a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. § 101 is clearly shown."). Further, the Federal Circuit has recently stated with respect to the rejection of claims for lack of utility that:

> The PTO cannot make this type of rejection . . . unless it has reason to doubt the objective truth of the statements contained in the written description. *See Brana*, 51 F.3d at 1566, 34 USPQ2d at 1441 ("[T]he PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.") (citations omitted); *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) . . . . The PTO may establish a reason to doubt an invention's asserted utility when the written description "suggest[s] an inherently unbelievable undertaking or involve[s] implausible scientific principles." *Brana*, 51 F.3d at 1566, 34 USPQ2d at 1441; *see also In re Eltgroth*, 419 F.2d 918, 164 USPQ 221 (CCPA 1970) (control of aging process).

In re Cortright, 49 U.S.P.Q.2d 1464, 1466 (Fed. Cir. 1999). Thus, the initial burden is on the Examiner to establish why one of ordinary skill in the art would *reasonably doubt* Applicants' assertions regarding utility. As discussed below, Applicants submit that the Examiner has not met the necessary burden to establish and maintain a rejection of the claims for lack of utility under 35 U.S.C. § 101.

Applicants further note that the "Revised Interim Utility Guidelines Training Materials" (Utility Guidelines) state that, "[t]he examiner should determine whether any asserted utility is specific and substantial, and if so, whether such utility is credible." Utility Guidelines, page 3. The Utility Guidelines also provide as follows:

> "Credible utility" - Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong". Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). *An assertion is credible unless* (A) the logic underlying

> the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

Utility Guidelines, page 5 (emphasis added). As explained below, the Examiner has provided no evidence that (1) the logic underlying Applicants' assertions of utility is seriously flawed or (2) the facts upon which Applicants base the assertions of utility are inconsistent with the logic underlying the assertions.

The Examiner asserts that "[t]he Examiner is under no obligation to prove a negative, the Examiner must simply provide sound reasoning in support of a conclusion that an element is lacking from a specification, and this has been done." *See,* Paper No. 23, Page 5, Paragraph 5. As Applicants have stated, the burden is on the Examiner to show that "one of ordinary skill in the art would reasonably doubt the asserted utility" *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). The Examiner has not fulfilled this burden. The Examiner has challenged the reasonability of the asserted utility based on homology. Applicants assert to the contrary, that in light of the homology, the skilled artisan would not doubt the asserted utility. Thus, Applicants respectfully urge that this challenge is unfounded.

The Examiner alleges:

> Applicant argues that "sequence relatedness or homology has, and is being used by those of skill in the art to predict function based on sequence" and cites Dunwell et al. in support of this position. However, a fair reading of Dunwell et al., does not lead to the conclusion that function can be predicted from structure. Even the title of Dunwell, "Microbial Relatives of the Seed Storage Proteins of Higher Plants: Conservation of Structure and Diversification of Function during Evolution..." suggests that conservation of structure is not predictive of function. The rejection of the instant claims was premised on the fact that proteins which are members of the CCN protein family share common amino acid sequence identity, but do not share common biological functions, therefore, one of ordinary skill in the art would not be able to predict which biological function would be possessed by a protein with a similar amino acid sequence. As stated by Dunwell, "simple analysis of primary sequence provides no information about the secondary or tertiary structure of the protein(s) under investigation, and it is the structure of a protein that determines its function" (see introduction). Therefore, Applicant's statement that "prediction of VIGF activity based on a shared percent identity of 40-45% with the CCN family in the instant specification would be found credible by those skilled in the art" has no basis in fact when Dunwell expressly states that primary sequence provides no information as to "structure" which determines function.

See, Paper 24, Pages 3-4, Paragraph 5.

The citation from Dunwell et al. relied upon by the Examiner reads in context as follows:

> The power of these analyses (mostly dependent on algorithms designed to detect similarities in gene or protein sequences) lies in their ability to identify similarity in the many million sequences now held in the major databases. However, despite the undoubted efficiency of these comparative studies, there remain several constraints, which limit the value of any new information that can be generated. First, each algorithm depends upon a certain level of similarity (usually above 30% identity) to detect a statistically valid relationship between two or more sequences. It is much more difficult, though not impossible, to confirm similarity where the degree of identity between sequences is 20% or lower. Second, simple analysis of primary sequence provides no information about the secondary or tertiary structure of the protein(s) under investigation, and it is the structure of a protein that determines its function. There is therefore a growing interest expanding from genome and transcriptome analysis into structural genomics and studies of the proteome and metabolome present in any specific cell or tissue.

See, Dunwell et al., Microbiol Mol Biol Rev, 64:153-179 (2000), at the introduction.

The above statement of Dunwell et al., points out the reasons they undertook their study, and the constraints of primary sequence analysis. With regard to which Dunwell et al. state:

> Figure 1 provides an alignment of a selection of putative cupin sequences arranged to show the two conserved motifs together with the increase in intermotif spacing from the basic value of 15 in many microbial enzymes up to 54, as found in a representative storage protein. It is acknowledged that absolute confirmation that all these sequences belong to the cupin family must await resolution of their tertiary structure, but in the meantime it is **reasonable** to propose this as a working hypothesis -- an approach supported by an independent study using PSI-BLAST.

See, Dunwell et al., *supra* at page 155, second column.

Thus, Dunwell et al., state that it is reasonable to assign a protein to a functional protein class based on the protein's primary amino acid sequence.

Further, the Examiner contends:

> Applicant cites Wilson et al. for stating that "40% sequence identity corresponds to a sharing of precise function while sequence identities of about 25% comprise a functional class". However, a fair reading of Wilson indicates that this is true of enzymes, and that this is not necessarily the case with nonenzymes in that "[t]here are differences

> between the functional conservation thresholds of enzymes and non-enzymes, with enzymes appearing to more highly conserve precise function than nonenzymes, but nonenzymes conserving functional class more highly that enzymes".

See, Paper 24, Page 4, Paragraph 5.

While Applicants acknowledge that Wilson et al. make this statement, Applicants point out that the conservation thresholds used by Wilson et al. were determined using both enzymes and nonenzymes in the analysis. Further, Wilson et al. explicitly state:

> Practically, then, when one searches an uncharacterized ORF against known structures, if the ORF matches a structure with a good e-value or percent identity, then the curves presented here can be used to check how the functional and detailed structure annotation will transfer. For example, if an unknown ORF matches a PDB structure with an e-value of 0.001 and a percent identity of 30%, then one can be assured that it has the same fold (Brenner *et al.* 1998) and according to our analysis it has a two-thirds chance of having the same exact function.

See, Wilson et al., J Mol Biol 17:233-49 (2000) under "Practical Implications".

Wilson et al. do not distinguish between enzymes and nonenzymes with regard to the ultimate usefulness of their method.

Therefore, Applicants again submit that the prediction of VIGF activity, for example, to enhance endothelial cell or vascular smooth muscle cell proliferation, based on a shared percent identity of 40-45% with the CCN family in the instant specification would be found credible by those skilled in the art.

The Examiner further contends:

> Applicant argues at page 4 of the response that Lassale et al. confirmed VIGF as having endothelial and smooth muscle cell specific expression and that they also predict that VIGF participates in vascular cell biology and human lung physiology. This point is noted, however, it still does not provide a specific, substantial and credible utility for the claimed invention. Expression of the claimed polynucleotide in endothelial and smooth muscle cells does not equate to a specific, substantial and credible utility. The likelihood that the claimed polynucleotide encodes an inhibitor of vascular smooth muscle and endothelial cell proliferation is just as great as that it encodes a stimulator of proliferation. Additionally, the claimed polynucleotide may be specific for one cell type verses another, which cannot be predicted from its pattern of tissue expression. At present, the instant claims are drawn to a nucleic acid which encodes a protein of as yet undetermined function or biological significance.

See, Paper 24, Pages 4-5, Paragraph 5.

Applicants have set forth in the specification statements that clearly and fully describe the function of VIGF and explain why Applicants believe the invention is useful. For example, the specification explicitly teaches that VIGF has use, for example, to proliferate vascular smooth muscle and endothelial cells (*see, e.g.,* the instant specification at page 4, lines 11-15; and page 20, line 24 through page 21, line 6). Thus, the specification clearly teaches a specific substantial and credible assertion of utility of the disclosed polynucleotides (and the encoded polypeptides) as involved in the enhancement of growth of vascular smooth muscle and endothelial cells leading to the stimulation of angiogenesis (*see, e.g.,* page 19, lines 7-9, and Example 5 of the instant specification). The biological role and significance of VIGF polynucleotides (and the encoded polypeptides), as well as its specific and substantial utility, are clearly taught by the specification as originally filed. Applicants assert that such characterization is sufficient on its own to constitute a showing of utility.

With regard to the Examiner's assertion that "the instant specification provides no evidence supporting the claimed utility in that tissue expression patterns and amino acid sequence similarity are not sufficient evidence to conclude that the claimed invention could be used in the manner disclosed in the instant specification" (*see,* Paper No. 24, Page 6, Paragragh 5), Applicants emphasize that evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. *See,* M.P.E.P. § 2107.01 (VII) at 2100-33. The disclosed homology between VIGF and the CCN family of proteins is evidence of utility. Thus, the skilled artisan, upon reading the specification, considering the Dunwell et al. and Wilson et al. references in support of the prediction of VIGF activity based on the shared percent identity with the CCN family of proteins, together with the Lassale et al. confirmation of endothelial and smooth muscle cell specific expression, would reasonably conclude that the use of VIGF to enhance the growth of vascular smooth muscle and endothelial cells is more likely than not true.

Indeed, the specification further teaches explicitly that "the VIGF-mediated increase in angiogenesis would be beneficial to ischemic tissues and to collateral coronary development in the heart subsequent to coronary stenosis" (*see*, specification, at page 19, lines 9-12); and that antagonists of VIGF "may be employed to inhibit turmor neovascularization and the neointimal proliferation of smooth muscle cells prevalent in atherosclerosis and restenosis subsequent to balloon angioplasty (*see,* the specification, at page 22, lines 28-31). Accordingly, Applicants have contemplated and disclosed many therapeutic applications of VIGF, for example, to treat muscle wasting diseases, osteoporosis,

to aid in implant fixation, to stimulate wound healing or tissue regeneration, to promote angiogenesis and to proliferate vascular smooth muscle and endothelial cell production, and therapeutic applications of antagonists of VIGF, for example, inhibition of tumor neovascularization and neointimal proliferation of smooth muscle cells prevelent in atherosclerosis and restenosis subsequent to balloon angioplasty, consistent with the biological activity of VIGF. *See, e.g.*, specification, at page 4 lines 11-15; and page 22, lines 28-31.

Moreover, Bechard et al., have further characterized the Endothelial-cell-specific molecule 1 (ESM-1) described by Lassalle et al., as cited in Applicants' last response. Bechard et al. show that ESM-1 release from the vascular endothelium may be regulated during inflammation, and suggest that "ESM-1 might possibly represent an additional marker of the endothelium activation and have a functional role in pathological disorders involving the vascular endothelium." (*See, e.g.,* Bechard et al., J. Vasc Res 37:417-425 (2000), attached as Exhibit A, at page 424.)

Applicants submit that the above asserted utilities for VIGF are specific (the vast majority of proteins do not enhance growth of vascular smooth muscle and endothelial cells leading to stimulation of angiogenesis) and substantial ("the general rule [is] that the treatments of specific diseases or conditions meet the criteria of 35 U.S.C. § 101." (Revised Interim Utility Guidelines Training Materials, p. 6)). In addition, Applicants submit that these utilities are credible.

With regard to these asserted therapeutic activities, Applicants note that there is no need to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty or provide actual evidence of success in treating humans where such a utility is asserted. M.P.E.P. § 2107.02 (I) at 2100-[33-34]. All that is required of Applicants is that there be a reasonable correlation between the biological activity and the asserted utility. *See, Nelson v. Bowler*, 626 F.2d 853, 857 (C.C.P.A. 1980). Moreover, "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re Brana, 51* F.3d *1560, 1568* (Fed. Cir. 1995) (emphasis added).

Even assuming, *arguendo*, the Examiner has established a *prima facie* showing that the claimed invention lacks utility, Applicants respectfully submit that they have rebutted the

Examiner's showing by proffering sufficient evidence that one skilled in the art would conclude that the asserted utilities are more likely than not true. Applicants have directed the Examiner to the specification where clear and specific assertions are made of VIGF biological and therapeutic activity and provided experimental evidence confirming the asserted utilities.

In view of the above, Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101. The Examiner has failed to establish and maintain grounds as to why a rejection for lack of utility is proper. Accordingly, Applicants respectfully request that the rejection be withdrawn.

Rejections Under 35 U.S.C. § 112

The Examiner has also rejected claims 54-67, 75-92, 102-107, and 115-119, and 122-175 under 35 U.S.C. § 112, first paragraph, as allegedly failing to adequately teach how to use the instant invention for the reasons given with regard to the rejection of these claims under 35 U.S.C. § 101.

In particular, the Examiner contends that

> Applicant argues that the claimed nucleic acids have specific and substantial uses "to enhance the growth of vascular smooth muscle and endothelial cells leading to the stimulation of angiogenesis." This argument is not persuasive because there is no evidence of record that the claimed nucleic acids encode a protein with this biological activity, therefore, the disclosed utility is not credible. The claimed invention shares sequence identity to members of the CCN family, wherein the biological activities of the family members is divergent, including members which have stimulatory activity and members which have inhibitory activity. There is no evidence of record to support the disclosed utility, therefore, the disclosed utility is not credible.

See, Paper 24, Page 7, Paragraph 6.

Applicants respectfully disagree and traverse.

For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, Applicants submit that the claimed invention is supported by a specific and substantial or well-established utility, for example, to enhance the growth of vascular smooth muscle and endothelial cells leading to the stimulation of angiogenesis (*see*, e.g., page 19, lines 7-9, and Example 5). Moreover, this immediate and specific utility is explicitly taught in the specification as filed. The Examiner "should not impose a 35 U.S.C. § 112, first paragraph,

rejection grounded on a "lack of utility" basis unless a 35 U.S.C. § 101 rejection is proper." M.P.E.P. § 2107(IV) at 2100-28 (Rev.1, Feb. 2000). Therefore, since the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejection of claims under 35 U.S.C. § 112, first paragraph, based on lack of utility of the claimed invention, should be withdrawn.

The Examiner further contends:

> It is again noted that the claims are also directed to nucleic acid molecules comprising contiguous portions of SEQ ID NO:1. These claims encompass genomic DNA, for which the instant specification fails to provide a written description. Therefore, these claims are also directed to subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Applicant did not appear to traverse this ground of rejection, therefore, the rejection is being reiterated and maintained at this time.

See, Paper No. 24, Page 7, Paragraph 6.

Applicants respectfully disagree and traverse.

Applicants further respectfully assert that they never intended to encompass naturally occurring genomic DNA within the claimed invention, nor would the skilled person find such a construction of the term "isolated polynucleotide" reasonable upon reading the specification. Applicants describe the use of the claimed polynucleotides, for example, as a diagnostic for detection of a mutated VIGF gene, or in gene mapping techniques such as fluorescence *in situ* hybridization with chromosomal spreads. *See, e.g.*, the specification at page 26, line 22 through page 29, line 29. These uses do not reasonably suggest that Applicants intended to claim genomic DNA that encodes VIGF, nor do the claims encompass such a scope.

Conclusion

In view of the foregoing remarks, Applicants believe they have fully addressed the Examiner's concerns and that this application is now in condition for allowance. An early notice to that effect is urged. A request is made to the Examiner to call the undersigned at the phone number provided below if any further action by Applicants would expedite allowance of this application.

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under

37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

Dated: MAY 15, 2001

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Characterization of the Secreted Form of Endothelial-Cell-Specific Molecule 1 by Specific Monoclonal Antibodies

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Cytokines · Vascular endothelium · ESM-1 · Inflammation · Sepsis

Abstract
Endothelial-cell-specific molecule 1 (ESM-1) is a recently identified endothelial cell molecule. As ESM-1 mRNA is preferentially expressed in human lung and kidney tissues, and as ESM-1 mRNA expression is regulated by inflammatory cytokines, ESM-1 is thought to play a role in the vascular contribution to organ-specific inflammation. In order to define its behavior, mouse anti-ESM-1 monoclonal antibodies were developed, and three distinct epitopes were mapped, which allowed development of a specific ELISA assay, immunohistological staining and immunoblot analysis. Here, we demonstrate that ESM-1 is present in cell lysates of human endothelial cells (human umbilical vein endothelial cells) with an apparent molecular weight of 20 kD. In contrast, the secreted form of ESM-1 is shifted to an apparent molecular weight of 50 kD, indicating that the secreted form of ESM-1 is posttranslationally modified. By ELISA, we show that the secretion of ESM-1 is significantly enhanced in the presence of TNFα. In contrast, the spontaneous as well as TNFα-induced secretion of ESM-1 is strongly inhibited by IFNγ. Moreover, ESM-1 was detected in the serum of healthy subjects at an average concentration of 1.08 ng/ml, and we demonstrated that the serum level of ESM-1 is dramatically increased in patients presenting a septic shock. Analysis of ESM-1 expression in normal human tissues by immunohistochemistry showed that ESM-1 is localized in the vascular network, but also in the bronchial and renal epithelia. Our results demonstrate that ESM-1 is mainly expressed in the vascular endothelium both in vitro and in vivo, but also by different epithelia. ESM-1 may represent a new marker of endothelial cell activation, and may have a functional role in endothelium-dependent pathological disorders.

Introduction

Endothelial cells form a multifunctional cell lining that covers all of the inner surface of blood vessels, and they regulate several important physiological and pathological reactions. The endothelium is a dynamic participant in cellular and organ function rather than a passive barrier as

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it was first believed. Through the expression of surface proteins and secretion of soluble mediators, the endothelium controls vascular tone and permeability, regulates coagulation and thrombosis, and directs the recruitment and homing of leukocytes into specific areas of inflammation [1]. This endothelial-cell-dependent control of leukocyte rolling, adhesion and transendothelial migration involves several known endothelial cell surface molecules including E- and P-selectins [2], ICAMs [3] and VCAM-1 [3], which are differentially regulated by proinflammatory cytokines like TNFα and IFNγ [4].

Recently, we described a new molecule called ESM-1 (endothelial-cell-specific molecule 1) and demonstrated that ESM-1 mRNA was restricted to endothelial cells and to human lung and kidney tissues [5]. ESM-1 mRNA levels were also shown to be regulated by cytokines. TNFα as well as IL-1β induced an increase in ESM-1 mRNA content in human umbilical vein endothelial cells (HUVECs) and IFNγ decreased it, suggesting a regulatory function during the inflammatory process. To better detect ESM-1, we developed and characterized a panel of anti-ESM-1 monoclonal antibodies allowing to analyze its behavior and to develop a specific detection method in human fluids and tissues. Besides, we demonstrated that ESM-1 was predominantly secreted as a 50-kD molecule from HUVECs. Secondly, we showed that potent inflammatory cytokines such as TNFα and IFNγ also act at the protein level and regulate the secretion of ESM-1 from HUVECs, and that IFNγ has an antagonistic effect on TNFα-induced secretion of ESM-1. Finally, we demonstrated that ESM-1 was detectable in the serum of healthy subjects and was highly increased in sera of patients presenting a systemic inflammatory syndrome such as septic shock.

Methods

Cell Culture

HUVECs and SV1 cells (derived from HUVECs by SV40 transformation) were cultured as described [6] and stimulated by TNFα (200 U/ml; Genzyme), IFNγ (1,000 U/ml; Genzyme) and IL-1β (10 U/ml; Genzyme).

Production of ESM-1 Full-Length Molecule and of ESM-1/Fc and ICAM-1/Fc Chimeras

The full-length ESM-1 cDNA was inserted into the eukaryotic expression vector pcDNA3 (Invitrogen) as previously described [5]. In order to select monoclonal antibodies raised against ESM-1, we generated two human Fc chimeras: ESM-1/Fc and a domain 1–3 ICAM1/Fc. The complete coding region of ESM-1 was flanked by two restriction sites (*Hin*dIII and *Eco*RI) generated by PCR, ligated in frame with the Fc domain of human IgG1 (Dr. R. Devos, Roche Research Gent, Gent, Belgium) and inserted as a *Hin*dIII-*Eag*I fragment into pcDNA3. In parallel, a control chimera that contained the first three domains of the human ICAM-1 in frame with the Fc domain of human IgG1 in pcDNA3 was engineered. The plasmids ESM-1/pcDNA3, ESM-1/Fc/pcDNA3 and ICAM1/Fc/pcDNA3 were then transfected in CHO and HEK293 cell lines with lipofectamine (Gibco BRL) to generate stable transfectants. The cells were cloned by limiting dilution in selective media (500 μg/ml G418; Gibco BRL). Cell supernatants were screened for the presence of ESM-1, ESM-1/Fc or ICAM-1/Fc secreted proteins by ELISA as follows. ELISA 96-well plates were coated overnight with an anti-human IgG Fc antibody (Sigma) at 5 μg/ml in carbonate buffer (0.1 *M* Na_2CO_3, 0.1 *M* $NaHCO_3$, pH 9.5). After a blocking step with 0.1% BSA in PBS, 5 m*M* EDTA, the wells were incubated sequentially with cell supernatant, followed by either ESM-1 polyclonal rabbit antibody [5] or ICAM-1 antibodies (Becton-Dickinson or 164B, ICOS corporation), then with either anti-rabbit or mouse secondary antibodies conjugated to horseradish peroxidase (Sigma). Finally, the plates were developed with OPD in substrate buffer (0.1 *M* Na_2HPO_4, 0.1 *M* NaH_2PO_4, 0.1 *M* citric acid, pH 5.5) containing 0.025% H_2O_2 and the reaction stopped with 4 *N* HCl solution. Each incubation was performed for 1 h at room temperature, and the plates washed between each step in ELISA buffer. Purified ESM-1/Fc immobilized with anti-human Fc antibodies bound polyclonal rabbit ESM-1 antibodies, this reactivity was abolished by preincubation of the coated plate with purified ESM-1, and the ESM-1/Fc chimera did not react with ICAM-1 antibodies demonstrating specific reactivity with the ESM-1 chimera. Clones producing the highest level of chimeras were selected.

Anti-ESM-1 Monoclonal Antibodies Generation

A C-terminal 14-kD ESM-1 peptide called Fo was produced in *Escherichia coli* as a glutathione-S-transferase (GST) fusion protein [5]. Three Balb/c mice were immunized with Fo (10 μg per mouse) and boosted 4 times in both feet. Mouse sera were tested in a sandwich ELISA for reactivity to ESM-1/Fc and ICAM-1/Fc chimeras. Three days after the last immunization, 10^8 cells were recovered from the inguinal lymph nodes of 1 mouse that was tested positive for the presence of anti-ESM-1 monoclonal antibodies (mAbs). The cells were fused with sp2/0 myeloma cell line by addition of PEG 1500 (Boehringer) as a recommended by the manufacturer. Hybridoma cells were then seeded into 10 96-well culture plates in RPMI-1640 with 10% FCS, hIL-6 (50 IU/ml; a generous gift from W. Fiers, Gent, Belgium), hypoxanthine, aminopterine and thymidine (Gibco BRL). Two weeks later, hybridoma supernatants were screened using the ELISA assay. Twenty-three clones that reacted with ESM-1/Fc and did not react with ICAM1/Fc were selected, subcloned and further characterized. The isotype of each mAb was determined by two commercial assays (Isostrip from Boehringer, and Mouse Immunoglobulin Isotyping Kit from Pharmingen). The clones were grown in a conditioned medium without FCS (CHO-SFM II; Gibco BRL) and cell supernatants passed through protein A (IgG2a) or protein G-sepharose (IgG1) columns (Pharmacia), washed with 20 m*M* Na_2HPO_4 (pH 7), eluted with 0.1 *M* glycin, HCl (pH 2.7) and immediately neutralized with 3 *M* Tris buffer. Purified mAbs were then dialyzed in PBS and concentrated through Centricon 30 (Amicon). The total protein amount was assessed with the Biorad protein assay, the purity by Coomassie-stained SDS-PAGE and ESM-1 reactivity by ELISA.

Epitope Mapping

In order to determine the ESM-1 epitopes recognized by mAbs, we generated 4 ESM-1 peptides with progressive deletions at the N terminus of the 105-amino-acid-long peptide used for mouse immunization. The cDNA were generated by PCR. The forward primers used are listed below:

FP1: 5′-TATGAATTCATGCAAAGACTGTCCCTACGGC
FP2: 5′-TATGAATTCAGGCATCTGTGACAGGGGGAC
FP3: 5′-ATAGAATTCAGTAACCAAGTCTTCCAACAGA
FP4: 5′-AAAGAATTCAGGCAATATTGTGAGAGAAGAA

The PCR fragments were inserted into the pGEX-4T3 vector (Pharmacia), expressed in *E. coli* as GST-ESM fusion proteins and purified on glutathione-sepharose as described by the manufacturer. The epitope mapping was performed by ELISA with the GST-ESM-1 fusion proteins coated on the plates. As controls we coated plates with GST, ESM-1/Fc, and ICAM-1/Fc molecules.

Quantitation of ESM-1 by ELISA

ESM-1 molecules were quantified using a sandwich immunoassay with MEP 19 (mAb against ESM-1 derived from prokaryotic cells; IgG1,K) and MEP 21 (IgG2a,K) that recognized respectively the antigenic determinant (AgD3 and AgD1) epitopes of ESM-1. MEP 19 mAbs were coated overnight in 96-well ELISA plates (0.5 µg/well in carbonate buffer; Costar), and then washed and blocked with saturation buffer. After a washing step, ESM-1 standards ranging from 100 to 0.5 ng/ml or samples were added. After an incubation and washing step, the complexes were incubated with MEP 21 mAbs, then washed and incubated with HRP-conjugated anti-mouse IgG2a (Pharmingen), washed again and developed with OPD as described. All washes and antibody dilutions were done in ELISA buffer (0.1% Tween 20 added to saturation buffer) except the dilutions of purified ESM-1 that were done in 10% FCS. All antibody incubations were done at room temperature for 1 h. The absorbance was determined at 492 nm on a spectrophotometer.

Characterization of ESM-1: Immunoprecipitation and Western Blot

The size of the ESM-1 was determined by immunoprecipitation combined with Western blots on cell lysates and supernatants. HUVECs were cultured as described above. For cell lysates, HUVECs were lysed in lysis buffer containing 0.5% NP40 anti-proteases cocktail (Boehringer) in PBS for 30 min at 4°C in agitation. Then, lysates were centrifuged at 10,000 *g* for 15 min in order to obtain cleared cell lysates. Supernatants were filtrated at 0.22 µm. In a further step, MEP 14 antibodies (2 µg) versus control antibodies (anti-ICAM-1; ICOS) were added to the cleared lysates or supernatants and incubated overnight at 4°C with agitation. The solution was then incubated with anti-mouse immunoglobulin conjugated to agarose beads (Sigma) at 4°C for 90 min, centrifuged, washed twice with lysis buffer and twice with PBS. The beads were resuspended in 20–40 µl of SDS-PAGE sample buffer for 5 min, boiled, centrifuged and the supernatants analyzed. Samples were migrated on SDS-PAGE and blotted onto nylon membranes according to standard procedures. After a blocking step, the membranes were incubated with MEP 19 and 21 antibodies at 1 µg/ml, washed and then incubated with an anti-mouse Fc HRP-conjugated secondary antibody (Sigma) followed by washes and developing using an ECL detection kit (Amersham). Antibody incubations were done for 1 h at room temperature.

Patients with Septic Shock

Serum levels of ESM-1 were measured in 20 healthy individuals (mean age = 44 years ± 9; 11 males and 9 females) and in 8 patients hospitalized for septic shock in the medical intensive care unit, CHRU Lille, France. The 8 patients had septic shock (mean age = 52 years ± 6; 4 males and 4 females) as defined by the consensus conference of the American College of Chest Physicians and Society of Critical Care Medicine [7]. Sera were collected at the time of intensive care unit admission. Serum concentrations of ESM-1 were measured by the specific ELISA here described.

Immunohistological and Immunocytological Staining

HUVECs were cultured in 8-well culture chamber slides (Lab-Tek; Falcon) for 24 h, then fixed with 4% PFA for 30 min, washed in PBS and stained with the ESM-1 mAbs using conventional APAAP protocol according to the published procedure [8]. Samples of human normal lung, kidney and gut tissues were taken from surgical specimens at a distance from the injured areas and either snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistological staining using biotin-conjugated anti-murine IgG antibody (Dako) and HRP streptavidine were performed according to the manufacturer's procedure (Sigma). Slides were counterstained with Gill's hematoxylin. The specificity of ESM-1 staining was determined by using two antibodies directed against different epitopes: MEP 08 (AgD2) and MEP 04 (AgD3) and by incubation with an isotype matched control.

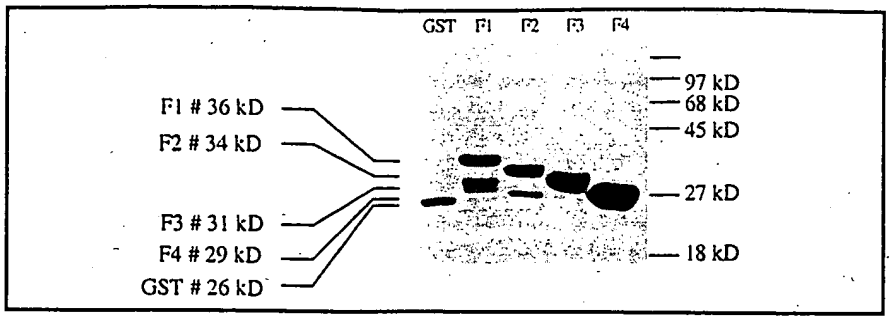
Statistics

Comparisons among treatment groups were performed using Wilcoxon's test. Analyses were performed using the Statview 5.0 statistical package. All reported p values of less than 0.05 were considered to indicate statistical significance.

Results

Generation and Epitope Mapping of Anti-ESM-1 mAbs

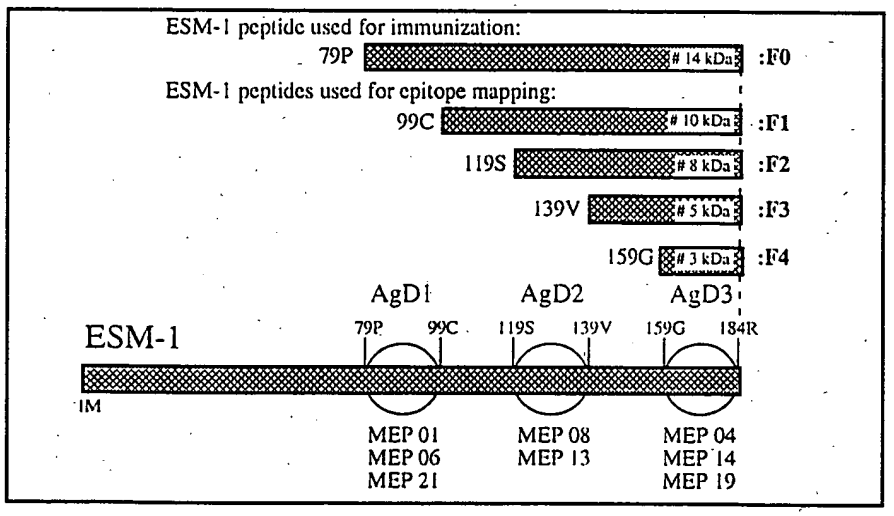
mAbs directed against ESM-1 were generated by immunization of mice with a C-terminal 14-kD ESM-1 peptide that was produced in *E. coli* as a GST fusion protein [5]. To screen hybridoma supernatants for anti-ESM-1 mAbs we generated ESM-1/Fc and ICAM-1/Fc chimeras and expressed them in eukaryotic cells. Twenty-three hybridoma clones designated MEP 01 to MEP 23 were shown to react with ESM-1/Fc and did not react with ICAM1/Fc. We identified the recognized antigenic determinant by the 23 mAbs by ELISA using 5 GST-ESM-1 fusion peptides: the 14-kD ESM-1 peptide (Fo) used for immunization plus 4 additional peptides (named respectively F1, F2, F3 and F4) with 20, 40, 60 or 80 amino acids progressively deleted from the N-terminal end of Fo as shown in figure 1A. The binding pattern of each antibody on the fusion peptides allowed to localize the ESM-1 epitope recognized by each mAb. All the mAbs reacted



A

Antigen:	ICAM1-Rg	ESM-Rg	GST	Fo	F1	F2	F3	F4	AgD
Balb/C	0,005	0,035	0,017	0,043	0,068	0,065	0,038	0,041	
MIS	0,012	2,720	0,161	2,502	2,662	2,665	2,520	2,510	
MEP 01	0,016	2,570	0.003	2,500	0,028	0,030	0,012	0,019	1
MEP 04	0,023	2,700	0.008	nd	2,770	2,800	2,700	2,700	3
MEP 06	0,045	2,590	0,011	2,580	0,024	0,025	0,010	0,020	1
MEP 08	0,076	2,800	0,010	nd	1,540	1,500	0,015	0,014	2
MEP 13	0,012	2,690	0,007	nd	2,850	2,900	0,035	0,070	2
MEP 14	0,034	3,000	0,010	nd	3,000	3,000	2,900	3,000	3
MEP 19	0,013	3,000	0,012	nd	3,000	3,000	3,000	3,000	3
MEP 21	0,008	2,860	0,015	2,630	0,057	0,032	0,020	0,020	1

B



C

Fig. 1. Characterization and epitope mapping of ESM-1 mAbs. **A** Coomassie-stained acrylamide gel of purified GST-ESM peptides used for epitope mapping. **B** ELISA data of anti-ESM-1 mAbs on plates coated with different ESM-1 peptides and controls. The data are given in absorbance. Fo = ESM-1 peptide 79P-184R; Balb/c = control serum from Balb/c; MIS = mouse immune serum against ESM-1. **C** Schematic representation of the AgD recognized by the ESM-1 mAbs within the ESM-1 molecule (one-letter codes for one amino acid).

with the fusion proteins and the positive ESM-1/Fc control, while none reacted with the negative control proteins ICAM-1/Fc and GST, demonstrating their specificity for ESM-1 (fig. 1B). Three groups of ESM-1 mAbs were identified and are reported in figure 1C. The first group of mAbs (AgD1) recognized the antigenic determinant of ESM-1 spanning from $Proline_{79}$ to $Cysteine_{99}$, the second group (AgD2) recognized the ESM-1 region spanning from $Serine_{119}$ to $Valine_{139}$, and the third group of antibodies (AgD3) recognized the region of ESM-1 spanning from $Glycine_{159}$ to $Arginine_{184}$. So, this mapping permitted to develop a sandwich ELISA in order to obtain a quantitative assay, which will be essential to evaluate ESM-1 levels in different biological fluids.

Immunoprecipitation and Western Blot Analysis of ESM-1

We analyzed the product of the ESM-1 gene expressed in HUVECs by immunoprecipitation and Western blot analysis. Distinct forms of ESM-1 were identified (fig. 2). In HUVECs lysates, but also in SV1 cell lysates (data not shown), ESM-1 appeared as two doublets of approximately 17 and 15 kD. This apparent molecular weight of 17 kD increased to nearly 20 kD under reducing conditions, consistent with its calculated molecular weight and internal disulfides. Interestingly, in the supernatants, a specific 50-kD form of ESM-1 was observed. No size reduction was obtained under reducing conditions, suggesting that ESM-1 was secreted by HUVECs in a posttranslational form. The mean production rate of secreted ESM-1 was established around 1 ng/24 h for ten millions of cells in RPMI medium supplemented with 20% FCS.

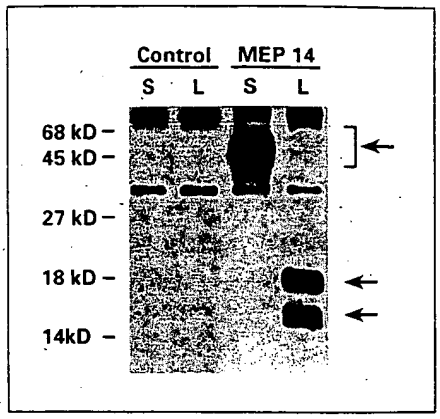


Fig. 2. Immunoblots of ESM-1 in HUVECs. Immunoprecipitation was performed with MEP 14 antibody, and immunoblot was probed with a combination of MEP 21 and MEP 19 anti-ESM-1 mAbs, each at 1 μg/ml, final dilution. The second HRP-labeled anti-mouse antibody was affinity purified, and yielded negative results when used alone. Arrows indicate the specific bands. Immunoprecipitations in HUVEC supernatants and lysates. S = supernatant; L = lysate.

Proinflammatory Cytokines Regulate the Secretion of ESM-1 by HUVECs

To study the regulatory pattern of ESM-1 by cytokines at the protein level, a sandwich immunoassay (ELISA) for ESM-1 has been developed. After addition of TNFα, the amounts of ESM-1 were increased in the cell supernatants at 24 h, reaching a plateau at 48 h (11.4 ng/ml ± 2.12 versus control: 4.59 ng/ml ± 0.92; fig. 3A). When HUVECs were stimulated with IFNγ, a slight decrease in the ESM-1 level in the supernatants, as compared to the controls, was observed at 48 h (2.26 ng/ml ± 0.44; fig. 3A). However, when combined with TNFα, the ability of IFNγ to inhibit the ESM-1 secretion was observed, resulting in a nearly complete inhibition of the TNFα-induced ESM-1 release at 48 h (4.28 ng/ml ± 0.82; fig. 3A).

As a control for cytokine stimulation, we also evaluated the levels of ICAM-1 expression in HUVECs with and without stimulation. Stimulation of HUVECs by TNFα or IFNγ alone induced a significant increase in ICAM-1 expression (fig. 3C). However, in contrast to its effect on ESM-1 expression (fig. 3B), IFNγ increased ICAM-1 expression significantly and had an additive effect in the presence of TNFα (fig. 3C). These results suggest that the secretion of ESM-1 by endothelial cells is under the control of inflammatory cytokines.

Serum Concentration of ESM-1 Is Increased in Septic Patients

To examine whether the production of ESM-1 in humans could be regulated in the context of acute inflammatory diseases, ESM-1 levels were measured in the sera of 8 patients suffering from septic shock and compared to 20 healthy subjects. As shown in figure 4, the median level of ESM-1 was 7.815 ng/ml (5.520–11.695) in the sera of septic patients, significantly higher than the median level in the sera of healthy subjects (1.081 ng/ml, 0.877–1.227). These results indicated that the soluble form of ESM-1 could be detected in the serum of humans and suggest that the vascular endothelial-derived secretion of ESM-1 may be up-regulated in vivo during an acute and severe inflammatory reaction in human beings.

ESM-1 Is Expressed both by the Vascular Endothelium and by Epithelia in vivo

In order to examine the distribution of ESM-1 in human tissues, preliminary immunocytochemical studies in HUVECs were performed. The staining was diffuse in the cytoplasm (fig. 5A). The results were similar using two mAbs recognizing distinct epitopes. No staining of membranes was observed, in contrast to ICAM-1, which was associated with the cell surface (data not shown). The specificity of the staining was demonstrated by the absence of staining with isotype-matched control antibodies.

ESM-1 was also detected by immunohistological staining first in endothelial cells, but also in different organs. In the lung, ESM-1 was expressed by venules, arterioles and alveolar capillaries and by epithelial cells of the bronchi and of the submucosal glands (fig. 5B). In kidney, ESM-1 was expressed in renal tubular epithelial cells at higher

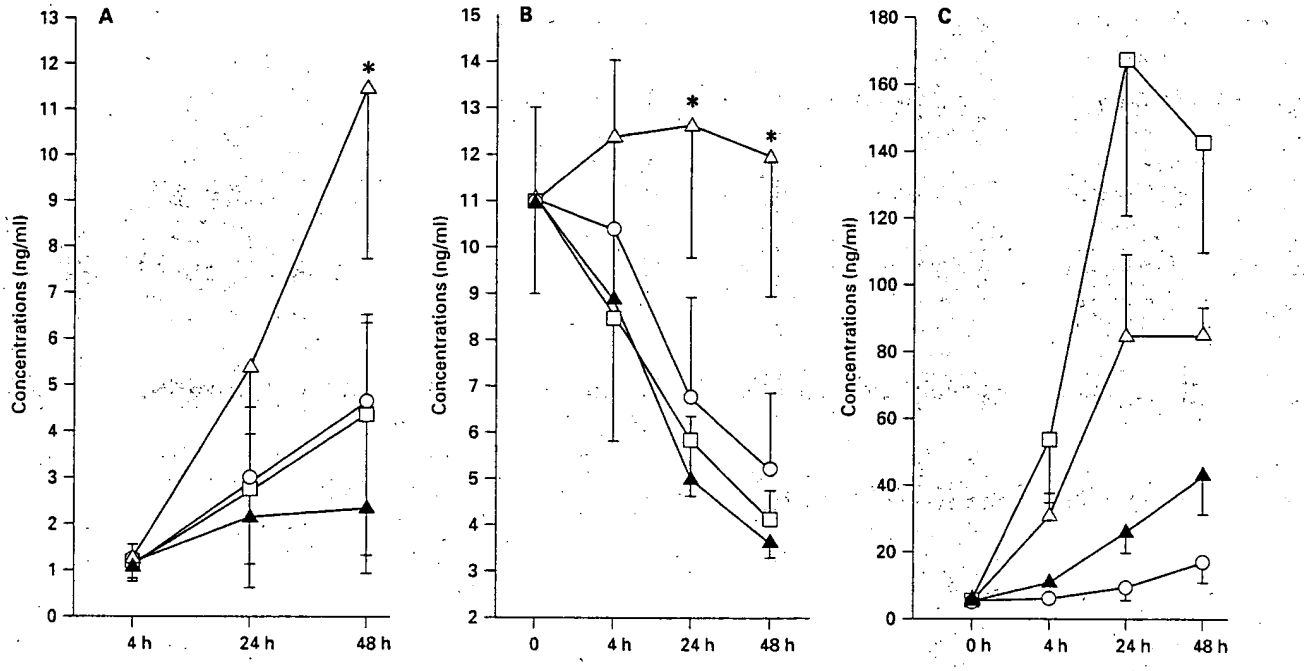


Fig. 3. Effects of inflammatory cytokines on ESM-1 expression in HUVECs. HUVECs were cultured in 6-well culture plates. At confluency, the cells were washed twice with RPMI-20% FCS medium and incubated overnight in the same medium. After 2 washings, the cytokines were added in 1 ml fresh medium: control medium (○); TNFα (200 IU/ml; △); IFNγ (1,000 IU/ml; ▲); TNFα + IFNγ (□). At the indicated times, the supernatants and the cell lysates were recovered and kept at −20°C until ESM-1 assay. The results are means ± SD of 5 experiments. * $p < 0.05$. **A** ESM-1 (ng/ml) in HUVEC supernatants. **B** ESM-1 (ng/ml) in HUVEC lysates. **C** ICAM-1 (ng/ml) in HUVEC lysates.

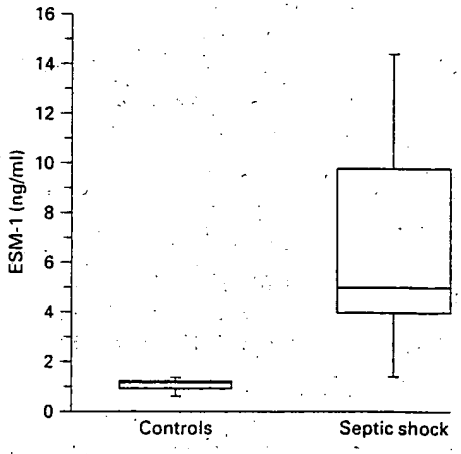


Fig. 4. Evaluation of the serum concentrations of ESM-1 of healthy subjects and of patients with septic shock. Concentrations of human soluble ESM-1 in the sera of healthy subjects (controls; n = 20) and of patients presenting a septic shock (n = 8) were evaluated by specific ELISA. The bottom, median and top lines of the box mark the 25th, 50th and 75th percentiles, respectively. The vertical line shows the range of values comprised between the 5th and the 95th percentiles. Median concentrations of ESM-1 were 1.081 ng/ml (0.877–1.227) in controls and 7.815 ng/ml (5.520–11.695) in patients with septic shock. The p values for comparison between controls and patients were 0.0173.

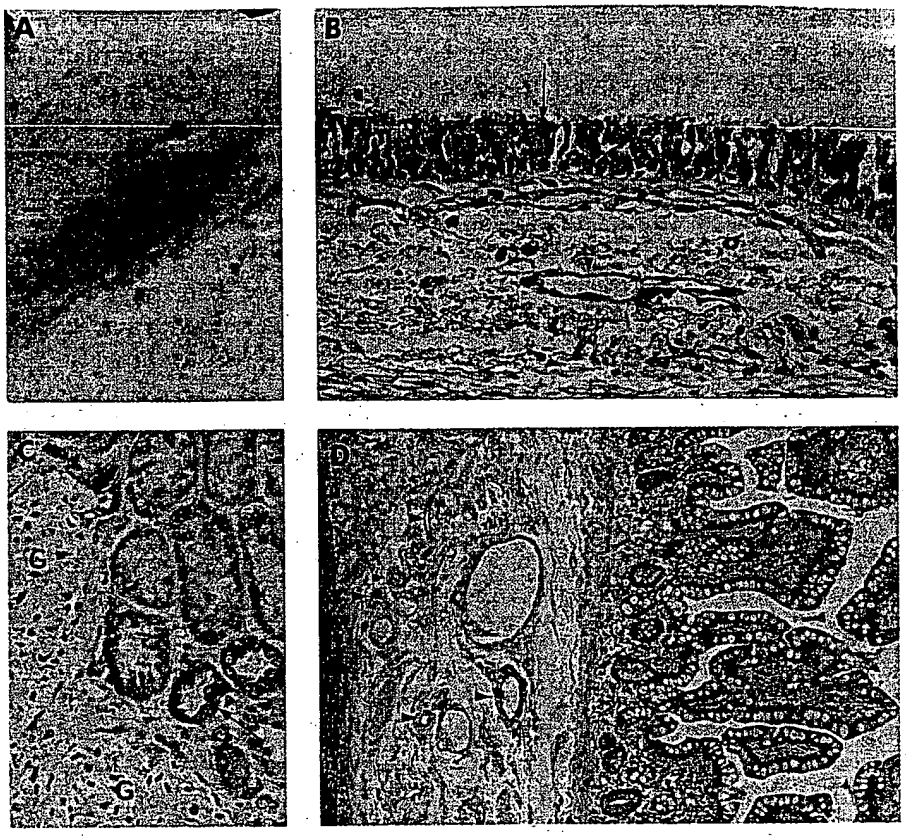


Fig. 5. Immunohistochemical staining in HUVECs, in normal human lung, kidney and gut. The specificity of ESM-1 staining was determined by using two antibodies raised against different epitopes, MEP 08 (AgD2) and MEP 04 (AgD3), and by incubation with an isotype-matched control. **A** Cytoplasmic immunostaining in HUVEC. HE. × 1,000. **B** Normal human lung. Expression in capillary endothelial cells (arrowhead) and by epithelial cells of the bronchi (arrow). HE. × 250. **C** Normal human kidney. Expression by tubular epithelial cells, higher expression in distal tubules (arrow). Lack of expression in glomerular (G) capillaries. HE. × 400. **D** Normal human gut. Expression in capillary endothelial cells (arrowheads). HE. × 100.

levels in the low epithelium of distal tubules and lower levels in the high epithelium of proximal tubules (fig. 5C). In addition, ESM-1 was also detected in the vascular endothelium in the kidney during human kidney allograft rejection (preliminary results). In the gut, ESM-1 was detected in capillaries and venules of the lamina propria (fig. 5D). These results were obtained with either MEP 04 and MEP 08, but not with the mAbs directed against the AgD1. So, ESM-1 was both detected in endothelial cells and in epithelial cells in normal lung, kidney and gut.

Discussion

The vascular endothelium is involved in many essential biological processes, and its dysregulation has important pathological consequences in the areas of hemostasis, angiogenesis, inflammatory recruitment and intercellular communications [1, 9]. Recently, we isolated the cDNA encoding for a polypeptide called ESM-1, whose expression was initially found to be restricted to the endothelial cells and to the lung and kidney tissues [5]. Consequently, it was essential to develop tools to characterize the expression of ESM-1. We generated 23 specific mAbs against the native form of human ESM-1, and mapped three recognized antigenic determinants allowing to obtain a quantitative biological immunoassay. In HUVEC lysates, the anti-ESM-1 mAbs immunoprecipitated the major cell-associated ESM-1 at 20 kD under reducing conditions. This apparent molecular weight was highly consistent with the predicted amino acid sequence and with the previously shown immunoprecipitated product from COS cells transfected by ESM-1 cDNA [5]. The reason for the presence of two doublets in Western blot is unknown. One could hypothesize that ESM-1 doublets would correspond to different states of phosphorylation, but ESM-1 could not be detected by anti-phosphothreonine, anti-phosphoserine or anti-phosphotyrosine mAbs in Western blot. Another hypothesis is the action of endogenous exopeptidases during the maturation process of ESM-1 polypeptide. In addition, a second cell-associated ESM-1 product was found, approximately 2 kD smaller. It may be the result of a peptide cleavage or indicate a splice variant of ESM-1. Nevertheless, this size reduction occurred specifi-

cally in HUVECs, and this smaller ESM-1 product was not observed in ESM-1-transfected COS cells.

In HUVEC supernatants, anti-ESM-1 mAbs immunoprecipitated the major ESM-1 form with an apparent molecular weight of 50 kD. Since no size reduction was observed under reducing conditions, the secreted form of ESM-1 is thought to be posttranslationally modified by O-glycosylation because no N-glycosylation site exists. Indeed, a recent work demonstrates that ESM-1 is secreted as a soluble chondroitin/dermatan proteoglycan [Bechard et al., submitted].

As the expression of ESM-1 was shown to be regulated at the mRNA level by inflammatory cytokines, and as ESM-1 is secreted by endothelial cells, we analyzed the effect of inflammatory cytokines on the secretion of ESM-1. TNFα enhanced consistently the secretion of ESM-1 by HUVECs, as has already been reported for IL-8 [10] and E-selectin [10]. On the contrary, IFNγ significantly decreased the secretion of ESM-1, as is known for the PECAM-1 expression [11], a specific component of EC junctions implicated in inflammatory recruitment. In addition, the unexpected inhibitory effect of IFNγ on the TNFα-induced ESM-1 secretion is consistent with its inhibitory effect at the mRNA level [5]. The inhibitory effect of IFNγ did not seem to be dependent of TNFα since IFNγ exhibited this effect when used alone. Combined with TNFα, IFNγ exhibited stronger inhibition on ESM-1 secretion, in clear contrast to the synergistic effect of these two cytokines on ICAM-1 expression [12] and RANTES secretion by endothelial cells [13]. Such an inhibitory effect of IFNγ is unusual, but has already been described for uPA [14] and IL-8 secretion [15] by HUVECs. Together, these findings suggest that ESM-1 and uPA genes may contain common regulatory elements involved in IFNγ-induced regulation in endothelial cells. Thus, these data indicate that ESM-1 release from the vascular endothelium may be regulated during inflammation. In order to approach the secretion of ESM-1 in vivo, preliminary experiments were carried out to detect ESM-1 in human serum samples. Indeed, ESM-1 is detected in human sera from healthy subjects. Interestingly, the mean level of ESM-1 in sera from patients with septic shock is significantly and consistently increased as compared to controls. This is in agreement with the idea that ESM-1 is released in vivo and that during acute inflammation, elevated serum ESM-1 level may reflect the degree of vascular cell activation induced by proinflammatory cytokines [9].

Finally, we investigated the vascular distribution of ESM-1 in different organs. ESM-1 was found to be expressed in many endothelial cells in normal tissues. It appears that it is a general property of the endothelial cells since most of the capillaries, arterioles and venules were similarly stained. In addition, epithelial cells in airways and kidney parenchyma were also labeled with the anti-ESM-1 mAbs. In the kidney, the epithelium of the distal tubule was strongly labeled, thereby indicating that the constitutive ESM-1 mRNA expression initially found in the kidney was derived both from the tubular epithelium and from vascular endothelial cells. The bipolar expression in both endothelia and epithelia is not exclusive to ESM-1. Indeed, several other molecules have been shown to be similarly distributed: endothelin [16] and ICAM-1 [2, 17] are examples of molecules expressed in both epithelia and endothelia, suggesting functional and/or embryologic relationships between these two cell types.

In conclusion, this work demonstrates that the major form of ESM-1 is secreted by HUVECs as a 50-kD molecule under the control of proinflammatory cytokines. In addition, we show a TNFα induction of ESM-1 release by HUVECs which is strongly antagonized by IFNγ, suggesting a fine regulation of ESM-1 secretion during inflammatory response. We also show that ESM-1 constitutively circulates in the bloodstream of healthy subjects and is dramatically increased during an acute inflammation such as septic shock. These data indicate that ESM-1 might possibly represent an additional marker of the endothelium activation and have a functional role in pathological disorders involving the vascular endothelium.

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